RECOMBINANT SENDAI VIRUS

Field of the Invention

The present invention relates to the recombinant Sendai virus and the method for preparing the same.

Background of the Invention

Sendai virus is also named hemagglutinating virus of Japan (HVJ), and classified in parainfluenza virus type I, belonging to the genus Paramyxovirus of the family Paramyxoviridae.

Sendai virus particle is pleomorphic, having the genome RNA without a function as template for translation (hereafter designated "negative strand RNA") enclosed in an envelope of 150-200 nm in diameter. Historically, Sendai virus has also been regarded as a biotechnologically useful virus, being widely utilized, especially for the production of heterokaryons and hybrid cells, by taking advantage of viral cell-fusion capacity. Also, Sendai virus-based cell fusing liposomes as a vehicle to deliver foreign genes into cells have been developed. Furthermore, Sendai virus is also used as the inducer for various interferons.

According to the classification based on the structure and polarity of genome nucleic acid, RNA viruses are classified into three groups, the double strand RNA viruses (dsRNA virus), positive strand RNA viruses, and negative strand RNA viruses. Sendai virus is a member of this third group (the negative strand RNA viruses). The dsRNA virus group includes reovirus, rotavirus, phytoreovirus, etc., and

have segmented, multipartite filamentous dsRNA genomes. Positive strand RNA viruses include poliovirus, Sindbis virus, Semliki forest virus, and Japanese encephalitis virus, which possess a single molecule of positive sense RNA as genome. The genome RNA can function as an mRNA and is capable of producing proteins required for viral RNA replication and particle formation. In other words, the genome RNA itself of positive strand RNA viruses is infectious and capable of disseminating.

In the present specification, by "disseminative capability (spreading capability)" is meant "the capability to form infectious particles or their equivalent complexes and successively disseminate them to other cells following the transfer of nucleic acid into host cells by infection or artificial techniques and the intracellular replication of said nucleic acid. Sindbis virus classified in positive strand RNA viruses and Sendai virus classified in negative strand RNA viruses have both infectivity and disseminative capability. On the other hand, adeno-associated virus classified in the parvovirus family has the infectivity but no disseminative capability (the mixed infection of adenovirus is necessary for the formation of disseminating viral particles). Furthermore, the positive strand RNA derived from Sindbis virus which is artificially transcribed *in vitro* is disseminative (to form infectious viral particles when transfected into cells). In contrast, not only genomic negative strand but also antigenomic positive

strand of Sendai viral RNA artificially transcribed *in vitro* cannot serve as a functional template to form infectious viral particles when transfected into cells.

Recently, viral vectors have been used as vehicles for gene therapy. In order to use them as gene therapy vectors, it is necessary to establish techniques for reconstituting viral particles. (By "reconstitution of viral particles" is meant the artificial formation of viral genome nucleic acid and the production of original or recombinant viruses in vitro or intracellularly.) This is because, in order to transfer foreign genes into viral vectors, viral particles should be reconstituted from the viral genome with foreign genes integrated by gene manipulation. Once techniques of viral reconstitution are established, it becomes possible to produce viruses with a desired foreign gene introduced, or with desired viral genes deleted or inactivated.

Also, once the viral reconstitution system is constructed and the viral gene manipulation becomes possible, said system appears to become a potential tool for genetically analyzing the viral function. Genetic analysis of viral functions is very important from the medical viewpoint of prevention and therapy of diseases etc. For example, if the replication mechanism of viral nucleic acid is elucidated, by utilizing the differences between said viral metabolism and host-cellular metabolism, it may be possible to develop viricide acting on the viral nucleic replication process and less damaging to host cells. Also,

by elucidating functions of viral gene-encoded proteins, it may become possible to develop antiviral drugs targeting proteins related with the viral infectivity and particle formation. Furthermore, by modifying genes concerned with the membrane fusion and preparing liposomes with superior membrane-fusing capability, it will be able to use them as gene therapy vectors. In addition, as represented by the interferon, the viral infection may induce the activation of host genes for viral resistance, resulting in the enhanced viral resistance of hosts. Genetic analysis of virus functions may provide more important information on the activation of host genes.

Reconstitution of DNA viruses possessing DNA as the genomic nucleic acid has been performed for some time, and can be carried out by the introduction of the purified genome itself, such as SV40, into monkey cells [J. Exp. Cell Res., 43, 415-425 (1983)]. Reconstitution of RNA viruses containing an RNA genome has been preceded by positive strand RNA viruses due to the dual function of these genomes as mRNA and the template for replication. For example, in the case of poliovirus, the disseminative capability of the purified genomic RNA itself was already demonstrated in 1959 [Journal of Experimental Medicine, 110, 65-89 (1959)]. Then, it was achieved to reconstitute poliovirus [Science, 214, 916-918 (1981)] and Semliki forest virus (SFV) [Journal of Virology, 65, 4107-4113 (1991)] by the introduction of cloned cDNAs into host cells, which encoded the respective

full-length plus strand viral RNAs.

The infectious cycle begins with the viral RNA synthesis from DNA, catalyzed by cellular DNA-dependent RNA polymerase. Furthermore, using these viral reconstitution techniques, gene therapy vectors have been developed [Bio/Technology, 11, 916-920 (1993); Nucleic Acids Research, 23, 1495-1501 (1995); Human Gene Therapy, 6, 1161-1167 (1995); Methods in Cell Biology, 43, 43-53 (1994); Methods in Cell Biology, 43, 55-78 (1994)].

However, as described above, in spite of many advantages of Sendai virus to be biotechnologically and industrially useful virus, its reconstitution system has not been established, because it is a negative-strand RNA. This is due to tremendous difficulty in reconstituting viral particles via viral cloned cDNA because neither genomic nor antigenomic RNA alone expressed from the cDNAs is active as the templates for mRNA synthesis and genome replication. This is absolutely different from the case of positive strand RNA viruses. Although, in JP-A-Hei 4-211377, "methods for preparing cDNAs corresponding to negative strand RNA viral genome and infectious negative strand RNA virus" are disclosed, the entire experiments of said documents described in "EMBO. J., 9, 379-384 (1990) were later found to be not reproducible, so that the authors themselves had to withdraw all the article contents [see EMBO J., 10, 3558 (1991)]. Therefore, it is obvious that techniques described in JP-A-Hei 4-211377 do not correspond to the related art of the

present invention. Reconstitution systems of negative strand RNA viruses were reported for influenza virus [Annu. Rev. Microbiol., 47, 765-790 (1993); Curr. Opin. Genet . Dev., 2, 77-81 (1992)]. Influenza virus is a negative strand RNA virus having eight-segmented genomes. According to these literatures, a foreign gene was first inserted into the cDNA of one of said genome segments, and then RNA transcribed from the cDNA containing the foreign gene was assembled with the virus-derived NP protein to form a ribonucleoprotein complex (RNP). Then, cells are transfected with the RNP and further infected with an intact influenza virus, in which the corresponding gene segment does not function under special pressure (such as the presence of neutralizing antibody and high temperature). In cells, gene reassortment occurs to generate a virus in which the genome segment is replaced with the above engineered segment to contain a foreign gene in a small population. This population is then selected and amplified under the pressure described above, thus ultimately generating a desired recombinant virus. Thereafter, the reconstitution of a nonsegmented negative strand RNA virus entirely from cDNA was reported for rabies virus belonging to the rhabdovirus family [EMBO J., 13, 4195-4202 (1994)].

Therefore, techniques for reconstituting negative strand viruses have become fundamentally known to the public. However, Sendai virus belongs to the Paramyxovirus family, defferent from the Rhabdovirus family. Sendai virus and rabies virus could differ in detailed mechanisms of gene

expression and replication. They also differ in protein components and virrion structure. Probably, for their reasons, the direct application of the above-described techniques for rabies virus did not support Sendai virus reconstitution. Also, the reconstitution of viral particles reported on the rhabdovirus was hardly detectable by routine virological procedures such as plaque production on susceptible cell cultures. Furthermore, the yield was not satisfactorily high for practical applications. Besides, in order to provide factors required for the viral reconstitution within host cells, helper viruses such as wild type viruses, recombinant vaccinia virus, etc. were conventionally introduced to host cells together with nucleic acids of the virus to be reconstituted. Accordingly, difficulties in separating the reconstituted desired virus from these harmful viruses were posing a difficult problem.

Summary of the Invention

An object of the present invention is to establish an efficient system for reconstituting Sendai virus, enabling the gene manipulation of Sendai virus, and providing Sendai viral vector sufficiently useful in the field of gene therapy, etc.

In order to apply to the reconstitution test of Sendai virus, the present inventors first made various investigations using cDNAs encoding the minigenome of Sendai virus. In this minigenome, the entire Sendai virus protein-coding sequence of ca 14 kb is replaced with a reporter gene

encoding the fire fly luciferase. This minigenome cDNA is flanked by T7 promoter and hepatitis delta virus ribozyme sequence in a circular plasmid. T7RNA polymerase encoded by a recombinant vaccinia virus was used to drive the plasmid in transfected cells. As a result, the inventors found efficient conditions regarding weight ratios among materials to be introduced into host cells, including the minigenome cDNA, the cDNAs encoding the nucleocapsid protein (N), the large protein (L), and the phosphoprotein (P) and minimizing cytotoxicity induced by the recombinant vaccinia virus to provide the T7RNA polymerase. The N protein derived from cDNA encapsidate the naked viral RNA derived from the minigenome cDNA to form the RNP, which is now active as the template for both viral mRNA synthesis and viral replication, which are also derived from the respective cDNAs. Furthermore, the present inventors obtained full-length cDNAs of both positive and negative strands, constructed plasmids to induce the intracellular biosynthesis of positive strand RNA (antigenome or cRNA) or negative strand RNA (genome or vRNA) of Sendai virus, and transferred said plasmid into host cells expressing N, P, and L proteins from the respective cotransfected plasmids. As a result, the inventors succeeded in reconstituting Sendai virus particles from cDNAs thereof.

That is, the present invention comprises the followings.

1. A recombinant Sendai virus having the genome with a desired foreign gene inserted or a desired viral gene deleted or inactivated, and retaining the disseminative capability.

2. The recombinant Sendai virus of description 1, wherein more than one gene encoding viral functional proteins are modified.

3. The recombinant Sendai virus of descriptions 1 or 2 possessing a foreign gene which can be expressed in host cells.

4. A vRNA molecule of the recombinant Sendai viruses of any one of descriptions 1-3.

5. A cRNA molecule of the recombinant Sendai viruses of any one of descriptions 1- 3.

6. A kit consisting of the following two components.

a. a DNA molecule comprising a template cDNA which can transcribe RNAs of descriptions 4 or 5, and

b. a unit capable of transcribing RNAs of descriptions 4 or 5 with said DNA as template *in vitro* or intrcellularly.

7. A kit consisting of the following two components.

a. a host expressing the NP, P, and L proteins of Sendai virus (each protein may be replaced with a protein having an equivalent activity), and

b. an RNA molecule of descriptions 4 or 5.

8. A method for producing the recombinant Sendai virus of descriptions 1-3, comprising introducing the RNA molecule of descriptions 4 or 5 into host cells expressing the NP, P, and L proteins of Sendai virus (each protein may be replaced by a protein having the equivalent activity).

9. A kit consisting of the following three components,

a. a host expressing the NP, P, and L proteins of Sendai

virus,

b. a DNA molecule comprising a template cDNA capable of transcribing RNAs or cRNAs of descriptions 4 or 5, and

c. a unit capable of transcribing vRNAs of descriptions 4 or 5 with said DNA as template in vitro or intracellularly.

10. A method for producing the recombinant Sendai virus of descriptions 1-3, comprising introducing the DNA molecule comprising a template cDNA capable of transcribing RNAs of descriptions 4 or 5, and a unit capable of transcribing RNAs of descriptions 4 or 5 with said DNA as template *in vitro* or intracellularly into hosts expressing the NP, P, and L proteins of Sendai virus.

11. A method for preparing foreign proteins comprising a process for infecting hosts with the recombinant Sendai virus of description 3, and recovering expressed foreign proteins.

12. A culture medium or allantoic fluid containing expressed foreign proteins obtainable by introducing the recombinant Sendai virus of description 3 into hosts and recovering said culture medium or allantoic fluid.

13. A DNA molecule realizing the expression of a protein encoded by a foreign gene integrated into a Sendai viral vector comprising said foreign gene inserted downstream of a promotor in an orientation for transcribing antisense RNA encoding said protein, and the said promotor.

Brief Description of the Drawings

Figure 1 is a schematic representation of plasmid pUC18/T7(+)HVJRz.DNA that generates antigenomic sense(+)

Sendai virus (HVJ) cRNA.

Figure 2 is a schematic representation of plasmid pUC18/T7(-)HVJRz.DNA that expresses genomic negative sense(-) vRNA of Sendai virus (HVJ).

Figure 3 is a graphic representation of the relationship between the postinfection time of CV-1 cells with SeVgp120 and HAU (the recombinant Sendai virus titer) as well as the expression level of the gp120 of human immunodeficiency virus type 1.

Detailed Description of the Invention

Sendai virus, the starting material in the present invention for the insertion of a desired foreign gene, or the deletion or inactivation of a desired gene may be a strain classified to parainfluenza virus type I, exemplified by Sendai virus Z strain or Fushimi strain. Furthermore, incomplete viruses such as DI (defective interfering) particles, synthetic oligonucleotides, etc. may be used partial materials.

Recombinant Sendai viral vectors of the present invention can be obtained, for example, by *in vitro* transcribing the recombinant cDNA encoding the gene-technologically produced recombinant Sendai viral vector genome, producing the recombinant Sendai viral genome RNA, and introducing said RNA to a host simultaneously expressing the NP, P, and L proteins (each protein may be a protein with an equivalent activity) of Sendai virus. Alternatively, Sendai viral vectors of the present invention can be obtained by introducing

a) the recombinant cDNA coding for the gene-technologically produced recombinant Sendai viral vector genome, and

b) a unit capable of intracellularly transcribing RNA with said DNA as template

into a host simultaneously expressing the NP, P, and L proteins (each protein may be a protein having an equivalent activity) of Sendai virus. In this case, said recombinant cDNA a) may be inserted downstream of a specific promotor, and said transcription unit b) may be a DNA molecule expressing a DNA-dependent RNA polymerase acting on said specific promotor.

Sendai virus particles can be reconstituted from its cDNA. The cDNAs introduced into host cells are more preferable in the circular form than in the linear form for the efficient reconstitution of viral particles. Not only the positive strand RNA but also the negative strand RNA can initiate highly successful reconstitution of viral particles although the former is superior to the latter.

Sendai virus reconstitution can be initiated following transfection with full-length viral RNA, either negative or positive sense, that has been synthesized *in vitro* from the cDNAs. This indicates that, if cells which express all viral proteins (N, P, and L) required for the initial transcription, replication, and encapsidation are constituted, the recombinant Sendai virus can be produced entirely without using helper viruses such as vaccinia virus.

Since cells expressing all the three viral proteins required for the initial transcription, replication, and encapsidation were already described [J. Virology, 68, 8413-8417 (1994)], those skilled in the art will be able to form such complementing cells. The cell type described in said reference is the one derived from the 293 cell line carrying three out of Sendai viral genes, namely N, P, and L on its chromosome, and expressing the proteins encoded by these three genes.

From numerous examples of viral vectors, if viral particles can be efficiently reconstructed from DNAs, it is obvious that those skilled in the art are able to readily exchange desired viral gene, insert a foreign gene, or inactivate or delete a desired viral gene. That is, it will be obvious to those skilled in the art that the first success in reconstituting Sendai viral particles by the present invention has enabled the gene manipulation of Sendai virus.

So far as the recombinant Sendai virus of the present invention maintain the disseminative capability, any foreign gene may be inserted at any site of RNA comprised in said recombinant, and any genome gene may be deleted or modified. Foreign genes to be inserted may be exemplified by genes encoding various cytokines and peptide hormones which can be expressed within hosts. In order to express the desired protein, the foreign gene encoding said desired protein is inserted. In the Sendai viral RNA, it is preferable to insert a sequence of a multiple of 6 nucleotides in length

between the sequences R1 (5'-AGGGTCAAAGT-3') SEQ ID NO:5 and R2 (5'-GTAAGAAAAA-3') SEQ ID NO:6 [Journal of Virology, Vol. 67, No. 8 (1993) p.4822-4830]. Levels of expression of a foreign gene inserted into a vector can be regulated by virtue of the site of gene insertion and the base sequences flanking said foreign gene. For example, in the case of Sendai viral RNA, it is known that there are increasing levels of expression of the inserted gene with decreasing distance of said gene from the promoter at the 3' terminus. Preferred hosts for expressing desired proteins may be any cells susceptible to the infection by the recombinant Sendai virus, exemplified by mammalian cells of various tissue-origin in culture and embryonated chicken eggs. It is possible to efficiently produce the foreign gene product by infecting these hosts with the recombinant Sendai virus integrated with expressible foreign gene and recovering the expressed foreign gene product. For example, proteins thus expressed can be recovered by the standard method from the culture medium when cultured cells are the host, and allantoic fluid when chicken eggs are the host.

When a foreign gene is inserted into a plasmid for expressing the negative strand Sendai viral RNA, it is necessary to insert said foreign gene downstream of the promoter in an orientation for transcribing an antisense RNA of said foreign gene encoding a protein. Such "a DNA molecule for expressing a protein encoded by a foreign gene integrated into a Sendai viral vector comprising the foreign

gene inserted downstream of the promotor in an antisense orientation for transcribing anisense RNA of said foreign gene encoding said protein and said promotor" has become available for the first time by the present invention, comprising a part of said invention.

Also, for example, in order to inactivate genes for immnogenicity, or enhance the efficiency of RNA transcription and replication, part of genes related with RNA replication of Sendai virus may be modified. Concretely, for example, at least one of the replication factors, the NP, P/C and L proteins may be modified to enhance or reduce the transcription and replication capabilities. The HN protein, one of the structural proteins, has dual activities as hemagglutinin and neuraminidase. For example, the reduction of the former activity may increase the viral stability in blood stream, and the modification of the latter activity may enable the regulation of viral infectivity. Also, the modification of the F protein mediating membrane fusion may be useful for improving membrane fusion liposomes constructed by fusing the reconstituted Sendai virus and artificial liposomes enclosing a desired drug or gene.

The present invention has enabled the introduction of point mutation and insertion at any sites of the genomic RNA, and is highly expected to accelerate the accumulation of genetic information on viral functions. For example, once the mechanism of viral RNA replication is elucidated, it may become possible to develop a viricide less harmful to a host

cell and targeting viral replication process by utilizing the differences between viral and cellular metabolisms. In addition, the elucidation of functions of viral gene-encoded proteins may contribute to the development of viricides targeting proteins involved in viral infectivity and reproduction. Concretely, for example, these techniques may be used for the analysis of antigen-presenting epitopes of the F and HN proteins which may act as antigenic molecules on the cell surface. Also, when a host cell gene for viral resistance is activated by viral infection, resulting in an elevated viral resistance, important information on such activation mechanism of host gene may be obtained by the genetic analysis of viral functions. Since Sendai virus is effective in inducing interferons, it is used in various basic studies. By analyzing the genome region necessary for inducing interferons, it may be possible to produce a non-viral interferon inducer. Techniques of the present invention are useful for the development of vaccines. Live vaccines may be produced by inoculating the recombinant Sendai virus with attenuating mutations to embryonated chicken eggs, and can be tested in animals (mice) for protection against the wild-type Sendai virus. Information thus obtained may be applied to other negative strand viruses, such as measles virus and mumps virus, with high demand for live vaccines. Furthermore, the present invention has enabled the usage of the recombinant Sendai virus as vectors for the expression of any prophylactic antigen in

body and gene therapy since virus vectors of the present invention derived from Sendai virus are expected to be highly safe in the clinical application, not disseminative in many types of tissues without endogenous proteases required for activation of Sendai virus infectivity, and expected to be therapeutically effective with a relatively small dosage.

In the following, the present invention will be concretely described with reference to Examples, but is not limited to these examples.

Example 1. Preparation of Sendai virus cDNA plasmids, pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA

Plasmid pUC18/T7(-)HVJRz.DNA was constructed by inserting a DNA molecule comprising T7 RNA polymerase promotor, Sendai virus cDNA designed to be transcribed to the negative strand RNA and the ribozyme gene in this order into pUC18 vector. Also, plasmid pUC18/T7(+)HVJRz.DNA was constructed by inserting a DNA molecule comprising T7 RNA polymerase promotor, Sendai virus cDNA designed to be transcribed to the positive strand RNA and the ribozyme gene in this order into pUC18 vector. Constructions of pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA are shown in Figs. 1 and 2, respectively.

Example 2.Reconstitution experiment of Sendai virus from cDNA

LLC-MK2 cells (2×10^6) trypsinized in a usual manner were placed in a 60-mm diameter plastic dish, and incubated in MEM medium (MEM supplemented with 10% FBS) (10 ml) in a 5%

CO_2 atmosphere at 37℃ for 24 h. After removing the medium and washing with PBS (1 ml), a suspension of recombinant vaccinia virus vTF7-3 expressing T7 polymerase in PBS (0.1 ml) was added to the cells at the multiplicity of infection (moi) of 2. The dish was gently agitated every 15 min to thoroughly spread the viral solution for 1 h infection. After removing the viral solution and washing with PBS (1 ml), a medium containing cDNA, which was prepared as follows, was added to the dish.

Nucleic acids shown in Tables 1 and 2 (containing plasmids expressing factors required for the replication of Sendai virus, pGEM-L, pGEM-P, and pGEM-NP were placed in a 1.5-ml sampling tube, and adjusted to a total volume of 0.1 ml with HBS (Hepes buffered saline; 20 mM Hepes pH 7.4 containing 150 mM NaCl). In those tables, (-) and (+)cDNAs represent plasmids pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA, respectively, and /C and /L indicate that cDNA is introduced into cells in the circular form and linear form after digestion of those two plasmids with restriction enzyme MluI, respectively.

On the other hand, in a polystyrene tube were placed HBS (0.07 ml), DOTAP (Boehringer Mannheim) (0.03 ml). To this tube was added the nucleic acid solution described above, and the mixture was left standing as such for 10 min. Then, to this mixture was added the cell culture medium described above (2 ml, MEM supplemented with 10% FBS) followed by the vaccinia virus inhibitors, rifampicin and cytosine

arabinoside C (C/Ara/C), to the final concentrations of 0.1 mg/ml and 0.04 mg/ml, respectively, resulting in the preparation of the medium containing cDNA described above.

The dish described above was incubated in a 5% CO_2 atmosphere at 37℃ for 40 h. The cells in the dish were harvested using a rubber policeman, transferred to an Eppendorf tube, sedimented by centrifuging at 6,000 rpm for 5 min, and re-suspended in PBS (1 ml). Aliquots of this cell suspension, as such or after diluted, were inoculated to 10-days old developing embryonated chicken eggs. That is, the cell suspension was diluted with PBS to the cell numbers shown in Table 1, and eggs inoculated with its 0.1 to 0.5-ml aliquots were incubated at 35℃ for 72 h, then at 4℃ overnight. Allantoic fluid was recovered as the source of reconstituted virus from these eggs using a syringe with a needle.

Hemagglutinin unit (HAU) and plaque forming unit (PFU) of the recovered virus solution were assayed as follows.

HAU was determined as follows. Chicken blood was centrifuged at 400 x g for 10 min and the supernatant was discarded. Precipitates thus obtained were suspended in 100 volumes of PBS, and centrifuged at 400 x g for 10 min to discard the supernatant. This procedure was repeated twice to prepare an 0.1% blood cell solution in PBS. Two-fold serial dilutions of virus solutions were prepared, and 0.05 ml each dilution to be assayed was dispensed into each well of 96-well titer plate. The blood cell solution (0.05 ml

each) was further added to each well, gently swirled to ensure a thorough mixing, and left at 4℃ for 40 min. The reciprocals of the highest virus dilution to cause the hemagglutination observable with the naked eye was taken as HAU.

PFU was assayed as follows. CV-1 cells were grown to a monolayer on a 6-well culture plate. After the culture medium was discarded, a virus solution 10-fold serially diluted (0.1 ml each) was dispensed into each well of the culture plate to infect the cells at 37℃ for 1 h. During the infection, a mixture of 2 x MEM free of serum and melted 2% agar (55℃) was prepared, and trypsin was added to the mixture to a final concentration of 0.0075 mg/ml. After 1 h infection and removal of the virus solution, the culture medium mixed with agar (3 ml each) was added to each well of the culture plate, and incubated under a 5% CO_2 atmosphere at 37℃ for 3 days. Phenol red (0.1%) (0.2 ml) was added to each well, incubated at 37℃ for 3 h, and then removed. Unstained plaques were counted to estimate the virus titer as PFU/ml.

Table 1 shows Sendai virus template cDNAs transfected into LLC-2 cells, amounts of cDNA factors, pGEM-L, pGEM-P, and pGEM-NP, required for the RNA replication incubation time, cell numbers inoculated to chicken eggs, HAU and PFU values recovered into the allantoic fluid.

Table 1

Template cDNA	amount (μg)	pGEM -L (μg)	pGEM -P (μg)	pGEM -NP (μg)	Incubation time (h)	Amount of cells	HAU	PFU
(+)cDNA/C	10	4	2	4	40	1.00×10^{5}	512	2×10^{9}
(+)cDNA/C	10	4	2	4	40	1.00×10^{5}	256	9×10^{8}
(+)cDNA/C	10	4	2	4	40	1.00×10^{6}	256	9×10^{8}
(+)cDNA/L	10	4	2	4	40	1.00×10^{5}	<2	<10
(+)cDNA/L	10	4	2	4	40	1.00×10^{5}	<2	<10
(+)cDNA/L	10	4	2	4	40	1.00×10^{6}	<2	<10
(-)cDNA/L	10	4	2	4	40	1.00×10^{4}	<2	<10
(-)cDNA/L	10	4	2	4	40	1.00×10^{5}	<2	<10
(-)cDNA/L	10	4	2	4	40	1.00×10^{6}	<2	<10
(-)cDNA/C	10	4	2	4	40	1.00×10^{4}	<2	<10
(-)cDNA/C	10	4	2	4	40	1.00×10^{5}	<2	<10
(-)cDNA/C	10	4	2	4	40	1.00×10^{6}	4	8×10^{3}

Samples showing both HAU and PFU were sedimented by ultra-centrifugation, re-suspended, purified by a sucrose density gradient centrifugation from 20% to 60%. The viral proteins of thus purified virions were fractionated by 12.5% SDS-PAGE. Each viral protein recovered from cDNAs samples was the same in size as that of the conventional Sendai virus.

These results demonstrated that Sendai virus can be reconstituted by introducing cDNAs into cells, and that virus particles are more efficiently reconstituted by introducing cDNAs transcribing positive strand RNAs as compared with those transcribing negative strand RNAs, and further by introducing cDNAs in the circular form rather in the linear form. The coexisting vaccinia virus in an amount of ca 10^4 PFU/ml in the allantoic fluid was readily eliminated by the virus once again in eggs at a dilution of 10^{-7} or 10^{-8}. This limiting dilution protocol was used to prepare vaccinia-free stock of recovered Sendai virus in this and all subsequent studies.

Example 3. Survey of RNA replication factors required for Sendai virus reconstitution

Experiments were performed to examine whether all three plasmids expressing the L, P, and NP proteins were required for the reconstitution of Sendai virus. Experimental methods were similar to those described in Example 2 except that any combinations of two out of pGEM-L, pGEM-P, and pGEM-NP plasmids or only one out of them, instead of all these three combined as in Example 2, were introduced together with a template cDNA into cells.

Table 2 shows Sendai virus template cDNAs introduced into LLC-MK2 cells, amounts of the cDNA plasmids required for RNA replication in trans, incubation time, number of cells inoculated into chicken eggs, and values of HAU and PFU.

Table 2

Template cDNA	amount (μg)	pGEM -L	pGEM -P	pGEM -NP	Incubation time (h)	Number of cells inoculated	HAU	PFU
(+)cDNA/C	10	4	2	4	40	$1.00x10^5$	256	$6x10^8$
(+)cDNA/C	10	4	2	4	40	$1.00x10^6$	512	$4x10^9$
(+)cDNA/C	10	0	2	4	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	0	2	4	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	0	4	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	0	4	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	2	0	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	2	0	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	0	0	4	40	$1.00x10^6$	<2	<10
(+)cDNA	10	0	0	4	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	0	2	0	40	$1.00x10^6$	<2	<10
(+)cDNA/c	10	0	2	0	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	0	0	40	$1.00x10^6$	<2	<10
(+)cDNA/C	10	4	0	0	40	$1.00x10^6$	<2	<10

As shown in Table 2, no virus reconstitution was observed by introducing any combinations of two out of these three factors into cells, confirming the necessity of all three proteins L, P, and NP for the virus reconstitution.

Example 4. Reconstitution experiment of Sendai virus *in vitro* from transcribed RNAs

Since the reconstitution of Sendai virus from the functional cDNA clones was described in Example 2, it was further examined whether transcription products of said cDNAs *in vitro*, that is, v or (-)RNA and c or (+)RNA, can initiate and support similar reconstitution.

After the Sendai virus cDNA plasmids, pUC18/T7(-)HVJRz.DNA and pUC18/T7(+)HVJRz.DNA, were linearized with restriction enzyme MluI, using these DNAs as templates, RNA synthesis was performed *in vitro* with a purified T7 polymerase preparation (EPICENTRE TECHNOLOGIES: Ampliscribe T7 Transcription Kit). The method for synthesizing in vitro RNAs essentially followed the protocols provided with the kit. Using RNA products thus obtained in place of cDNAs in Example 2, similar experiments were performed, and the virus production was estimated by HA test. Results are shown in Table 3.

Table 3

Template cDNA	amount (μg)	pGEM-L (μg)	pGEM-P (μg)	pGEM-NP (μg)	Incubation time (h)	Number of cells inoculated	HAU	PFU
in vitro (-)RNA	10	4	2	4	40	1.00×10^6	512	2×10^9
in vitro (-)RNA	10	4	2	4	40	1.00×10^6	512	ND
in vitro (+)RNA	10	4	2	4	40	1.00×10^6	2	5×10^3
in vitro (+)RNA	10	4	2	4	40	1.00×10^6	<2	ND

These results indicate that virus can be reconstituted by introducing either negative or positive sense strand RNAs into cells.

Example 5. Expression of foreign genes inserted into Sendai viral vectors in host cells

1. Preparation of Sendai virus vector "pSeVgp120" inserted with a foreign gene, the gp120 of human immunodeficiency virus type 2 (HIV)

Using a set of primers comprising primer a (5'-TGCGGCCGCCGTACGGTGGCAATGAGTGAAGGAGAAGT-3') (SEQ ID NO:1) and primer d (5'-TTGCGCCCGCGATGAACTTTCACCCTAAGTTTTTTATTACTACGGCG-TACGTCATCTTTTTTCTCTCTGC-3') (SEQ ID NO:2), the HIV-1gp120 gene was amplified on "pN1432" or a full-length cDNA of HIV-1

strain NL43 by the standard PCR techniques. PCR products were subjected to TA cloning, digested with NotI, and then inserted into the NotI site of "pSeV18$^+$". pSeV18$^+$ contains an additional 18 nucleotide sequence with a unique NotI restriction site which is placed before the ORF of NP gene of pUC/T7(+)HVJRz. Then, *E. coli* cells were transformed with this recombinant plasmid. DNAs were extracted from each colony of *E. coli* by the "Miniprep" method, digested with DraIII, and then electrophoresed. Positive clones (designated "clone 9" hereafter) were selected by confirming to contain DNA fragments of the size expected from the insertion. After DNA fragments were confirmed to have the authentic nucleotide sequence, DNAs were purified by a cesium chloride density gradient centrifugation. pSeV18$^+$ inserted with the gp120 gene is designated "pSeVgp120" hereafter.

2. Reconstitution of Sendai virus containing pSeVgp120 (SeVgp120) and analysis of gp120 expression

Reconstitution of the virus from pSeVgp120 in LLCMK2 cells, the virus recovery from allantoic fluid of embryonated chicken eggs, and assay of the viral HAU were done exactly as described in Example 2. The recovered virus was also examined for the expression of gp120 by ELISA as follows.

Samples (100 μl each allantoic fluid) were dispensed into each well of a 96-well plate which had been coated with monoclonal antibody against HIV-1, and incubated at 37℃ for 60 min. After washing with PBS, HRP-linked anti-HIV-1 antibody (100 μl each) was added to each well, and incubated

at 37℃ for 60 min. After washing with PBS, tetramethylbenzidine was added to each well, and amounts of reaction product converted by the action of HRP under acidic conditions were determined by following the optical density at 450 nm to estimate the expression amount of gp120. Results are shown in the left-hand column in Table 4.

The virus solution thus obtained was inoculated to CV-1 cells, and similarly examined for gp120 expression as follows. CV-1 cells were dispensed to a culture plate at 5 x 10^5 cells/plate, grown, and then the culture medium was discarded. After washing with PBS(-), the viral solution was added to the cells at the multiplicity of infection of 10, and incubated at 37℃ for 1 h. After the virus solution was discarded, washed with PBS(-), a plain MEM medium (MEM medium supplemented with antibiotics AraC and Rif, and trypsin) was added to the cells, and incubated at 37℃ for 48 h. After the reaction, the medium was recovered and assayed for HAU (by a similar method as described in Example 2) and examined for the expression of gp120 (by ELISA). Results are shown in the center column of Table 4. In addition, the supernatant of CV-1 cell culture medium was inoculated to embryonated chicken eggs again, and the virus solution thus obtained was assayed for HAU and also examined for the gp120 expression (by ELISA). Results are shown in the right hand column of Table 4.

Table4

(μg/ml)

Allantoic fluid (F1) gp120 (HAU)	CV-1 medium (F1) gp120 (HAU)	Allantoic fluid (F2) gp120 (HAU)
0.10 (4)	3.46 (128)	
0.15 (32)	1.81 (128)	1.56, 1.21 (512, 512)
0.05 (32)	2.20 (128)	

As shown in Table 4, markedly high concentrations of gp120 were detected in CV-1 cells in culture (center column of the Table), and also in the allantoic fluids from embryonated chicken eggs inoculated again with the virus (right-hand column of the Table). In the left-hand and center columns of the Table are shown the mean values of three clones.

Furthermore, the expression of gp120 was analyzed by Western blotting. After the culture medium of CV-1 cells infected with SeVgp120 was centrifuged at 20,000 rpm for 1 h to sediment virus, the supernatant was treated with either TCA (10%, v/v) for 15 min on ice or 70% ethanol at -20℃, and centrifuged at 15,000 rpm for 15 min. Proteins thus precipitated were solved in an "SDS-PAGE sample buffer" (Daiichi Chemicals) at 90℃ for 3 min, and then subjected to electrophoresis on 10% SDS-polyacrylamide gel (SDS-PAGE).

Proteins thus fractionated were transferred to PVDF membranes (Daiichi Chemicals), reacted with monoclonal antibody 902 at room temperature for 1 h, and then washed with T-TBS. The membranes were reacted with anti-mIgG (Amersham) at room temperature for 1 h, and washed with T-TBS,. The membranes were then reacted with HRP-linked protein A (Amersham) at room temperature for 1 h, washed with T-TBS, and 4-chloro-1-naphthol (4CNPlus) (Daiichi Chemicals) was added to detect gp120. As a result, protein bands were visualized at positions corresponding to the expected molecular weight of gp120.

In addition, effects of postinfection time of CV-1 cells transfected with SeVgp120 on the HAU value and gp120 expression amount were analyzed. CV-1 cells (5×10^6) dispensed to 10-cm plate were infected with SeVgp120 at the multiplicity of infection of 10, and the culture medium (1 ml each) was postinfectionally recovered at 30, 43, 53 and 70 h, mixed with an equal volume of the fresh medium, and subjected to HAU assay, gp120 expression examination (by ELISA) and Western blotting. Results are shown in Figure 4. As clearly shown in Fig. 3, the production of gp120 tends to increase with the increasing HA titer of Sendai virus.

Example 6. Analyses of SeVgp120 propagation and gp120 production in various types of cells

Using similar methods as those in Example 5 except for the use of various types of cells, HAU and gp120 expression levels (by ELISA) were assayed. Results are shown in Table

5.

Table 5

Cell type	Hours (postinfection)	HAU	rgp120 (μg/ml)
CV-1	96	32	2.5
LLCMK2	48	16	0.5
CHO	55	4	0.46
NIH3T3	48	4	0.25
MT4	24	16	0.8
MOLT4/	24	16	1.2

In the left-hand column of the Table are shown the postinfection times (hours) of various types of cells transfected with SeVgp120. As a result, SeVgp120 propagation and gp120 expression were detected in all types of cells tested.

Example 7. Studies on the expression of luciferase gene inserted into the Sendai viral vector in host cells

In order to isolate the luciferase gene for inserting to vectors, the luciferase gene bounded by the engineered NotI sites on both termini was constructed by the standard PCR using a set of primers [5'-AAGCGGCCGCCAAAGTTCACGATGGAAGAC-3') (30mer) (SEQ ID NO: 3)] and [5'-TGCGGCCGCGATGAACTTTCACCC-TAAGTTTTTCTTACTACGGATTATTACAATTTGGACTTTCCGCCC-3' (69mer) (SEQ ID NO: 4) with the minigenome encoding plasmid, "pHvluciRT4", as a template. The PCR product was cloned into the NotI window of pSeV18$^+$ to obtain a recombinant Sendai virus vector

to which the luciferase gene is inserted. Then, this recombinant vector was transfected into LLCMK2 cells, and after 3 cycles of freezing and thawing, the cells were inoculated into embryonated chicken eggs. Allantoic membranes of developing eggs were excised out, twice washed with cold PBS(-), and, after the addition of lysis buffer (Picagene WAKO) (25 μl) and thorough mixing, centrifuged at 15,000 rpm for 2 min. To the supernatant (5 μl each) was added the substrate (IATRON) (50 μl), and the mixture was dispensed into each well of a 96-well plate. Fluorescent intensity was measured with a luminometer (Luminous CT-9000D, DIA-IATRON), and the enzyme activity was expressed as counts per second (CPS). As a result, an extremely high luciferase activity was detected. The egg grown recombinant virus was purified by passaging once again in eggs, so that the stock virus did not contain helper vaccinia virus. This stock virus was then used to infect CV-1 cells and examine luciferase expression in these cells. As shown in Table 6, again, extremely high luciferase activity was detected for infected CV-1 cells at 24-h postinfection (Table 6). In these experiments, Sendai virus which did not carry the luciferase gene was used as control (represented by "SeV" in the table). Results obtained from two clones are shown in the table.

Table 6

	Fluorescence intensity (counts/10 sec)	
	Allantoic membrane	CV-1 (24h postinfection)
Luc/SeV	669187	
	2891560	8707815
SeV	69	48
	23	49

By the present invention, a system for efficient reconstitution of viral particles from Sendai viral cDNAs has been established, enabling the gene manipulation of Sendai virus to produce the recombinant Sendai virus comprising a genome with a desired foreign gene inserted or a desired gene deleted or inactivated, but retaining the disseminative capability.